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PATENT

Navy Case No. 84,622
Application No. 09/864,373

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	
Krise, et al.	
Serial No. 09/864,373	Group Art Unit: 1641
Filed: May 25, 2001	
For: MOLECULAR TAG READER	Examiner: Kartic Padmanabhan

DECLARATION UNDER RULE 1.131

I, William F. Krise, do hereby declare and say as follows:

Being a listed inventor in the above referenced patent application, I am extremely familiar with the invention and the development dates associated with said invention.

The invention claimed in the above referenced application was physically reduced to practice between September 1998 and March 1999 as indicated in the attached copy of an invention disclosure which was signed by myself and my co-inventor and witnessed in July 1999.

All statements made herein are of my own knowledge are true and that all statements made on information and belief are believed to be true; and, further, I understand that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

William F. Krise Date: April 13, 2004

William F. Krise

MAR 6

Form 9: Report of Invention Form DI-1215

Form DI-1215
(June 1965)

SUR 3657
For Solicitor's Office Use

UNITED STATES DEPARTMENT OF THE INTERIOR
REPORT OF INVENTION

(Prepare in triplicate)

This report is an important legal document, and should be read carefully before filling in data. The report and memoranda or correspondence concerning it are to be considered as confidential documents. Where necessary, use additional sheets to complete entries, identify with specific item designations as indicated on this form, and attach.

Date .....

I. INVENTOR'S IDENTIFICATION (1). (If there are more than two inventors attach information on additional sheet.)

A. Full name (including middle name or initial)	Citizenship	B. Residence address
John L. Sternick	U.S.	55 Wakefield Terrace Mansfield, PA 16933
C. Complete name of organization (including, as applicable, agency, section, branch, division, etc.) Mansfield University		D. Office address Biology Dept., Grant Science Center Mansfield, PA 16933
E. Position or title Associate Professor, Ph.D.		F. Official working place address Biology Dept., Grant Science Center Mansfield, PA 16933

INVENTOR'S IDENTIFICATION (2).

A. Full name (including middle name or initial)	Citizenship	B. Residence address
William F. Krise	U.S.	9 Riberolle Street Wellsboro, PA 16901
C. Complete name of organization (including, as applicable, agency, section, branch, division, etc.) USGS-BRD; Research & Development Lab.		D. Office address RR #4, Box 63 Wellsboro, PA 16901
E. Position or title Fishery Biologist, PH.D.		F. Official working place address RR #4, Box 63 Wellsboro, PA 16901

II. IDENTIFICATION OF THE INVENTION.

Title of the invention (Title should be brief but descriptive of the invention.)

Molecular Tag Reader

III. PROBABLE UTILIZATION OF THE INVENTION.

A. Give your opinion of the extent to which the invention may be used by any agency of the Department, other Government agencies, and the public.

The invention will provide government and private fishery management programs with a tool to rapidly identify stocks of fish or other wildlife without endangering them.(cont.

B. Discuss briefly the Government's interest, if any, in further developing the invention.

The device is a hand-held portable mechanical electro-optical system capable of rapidly reading small quantities of selected molecular tags in tissues of fish or other (cont.)

IV. DETAILED TECHNICAL DESCRIPTION OF THE INVENTION. (This description should be concise and include the following.)

A. The principal utility and the general field of application of the invention.

This invention is designed to provide quick and easy identification of marked fish; whole live animals or blood samples can be screened for identification using this tool. Fish are marked with a protein coupled to a laser dye with specific excitation and emission characteristics not found in the natural habitat. (cont.)

B. Briefly, what prompted the making of the invention, or how was the idea therefor obtained?

The making of the invention was prompted by the need to have a device that could be used in the field environment to identify fish or other animals rapidly and accurately as to their age, origin, and experimental protocols they may have been subject to. Previous methods are cumbersome (wire tags), lethal (otolith marks and wire tags), expensive, and technically demanding (rare earth metals and genetic testing); and all are time consuming. Most are to large to use with very small fish. (cont.)

Continued from III. A.

The invention will enhance the tracking of fish and wildlife more effectively at less cost.

Continued from III. B.

animals in the field. The invention can also potentially be used for commercial livestock herd identification and replace branding or tattooing of valued animals such as horses and dogs.

Continued from IV.A.

The protein and the dye are harmless to the host animal and its predators including man.

The invention is composed of four components: 1) a light source, such as a laser diode, 2) a sample holder, 3) an optical system, made of a fiber optic lens and a bandpass filer, and 4) a photodiode detector coupled to an LCD (see Figs. 1-4). The optical system can be modified to eliminate the fiber optics and bandpass filter.

The laser may be replaced by a miniature light bulb coupled to an extra bandpass filter to eliminate lower light wavelength that might interfere with the sensitivity of the instrument (see Fig. 2). The light sources and other systems can be battery operated.

The sample holder can be of two general types: 1) To hold fluids such as blood, serum, ore other body fluids for analysis (see Figs. 2-4). In this case the tubing that contains the sample has a small diameter to accommodate microliter samples. The sample is aspirated into the tubing to a specific spot with a stepper motor piston assembly, controlled by a motor control board (see Fig. 1). This specific spot is the position where the light hits the sample and the optical system reads the amount of light emitted from the sample. 2) To hold whole organisms for analysis, i.e.: marked eels (see Fig. 4) are subject to the same procedure as above where one position on the eel is analyzed for the presence of the tag. An alternative method, is to scan the whole eel. This procedure is accomplished by pulling the tube containing the eel through the sample holder past the light source and the optical system of the detector. If the tag is present at any location on or in the eel, the detector will pickup the emitting signal.

The optical system is optimized for the type of tag used in the marking procedure, for instance when the laser dye NN 382 is used as a tag, the light excitation (laser or light bulb) is set at the optimum emission wavelength, i.e., 778 nm and the detection is set for 806 nm which is the optimum emission wavelength of the dye. Other settings can be selected depending on the dyes used for tagging. This can be done by changing the laser and bandpass interference filters in the device. Multiple dyes in one organism can be tested by this invention by changing the excitation wavelength and emission measurement settings.

Continued from IV. B.

The advantages of the invention are as follows:

1) Speed of operation. It takes seconds versus minutes, hours, or days in the case of genetic analysis.
2) Uses small quantities of tissues or whole organism.
3) Non lethal.
4) Environmentally safe.
5) Sturdy, solid state, light weight, compact, easy to use device in the field by a non-skilled operator.
6) Precalibrated, no standard curve necessary.
7) Operates in the near infrared, tissues have low background in this range. This increases reading sensitivity.
8) Can be used under sterile or non-sterile conditions.
9) Compared to other methods is not expensive.
10) Cannot be removed by poachers.
11) Can be used to encode complex information over long periods of time (years).

C. Previously known or used methods, [redacted] devices performing the task or function of the [redacted], and the disadvantages of such prior art. In this connection discuss the particular problems encountered with the prior art. List all pertinent literature references and patents of which the inventor has knowledge.

We know of no same or similar devices previously used for this function.

D. Respond to this part on separate sheets as enclosures to be attached hereto.

Give explanation of a specific embodiment of the invention:

1. Include therein the theory of the operation of the invention.
2. In a mechanical or electrical invention give a detailed description by reference to a sketch or drawing. All component parts of the apparatus must be labeled and the description keyed thereto.
3. In a process or chemical composition, include the extreme and preferred ranges of conditions (e.g., temperature, pressure, ratio of components, voltage, current, etc.) and alternate or equivalent materials which may be employed.
4. Include any additional material such as photographs, reports, publications, and refer to texts or other informational material which may be helpful to an understanding of the invention.

E. Alternate embodiments of the invention including specific examples. To the extent found to be appropriate, follow the instructions given in regard to D above.

F. The advantages of the Invention over the prior art noted in connection with Item C.

We can mark very small fish, where there was no previous method, and detect that mark using this reader.

Other advantages are listed under IV. B.

G. Features of the Invention believed to be new.

These features include:

1) Detection of laser dye tagged molecules in vivo & in vitro with laser or other light sources for animal identification.
2) Reader can be hand-held, or used in field applications.
3) Reader can identify origin of animals marked when they are very small.

H. If this is believed to be a joint invention, the contribution of each inventor.

50%

Attached hereto and comprising a part of this disclosure are _____ supplemental pages.

CERTIFICATION OF INVENTOR(S)

I certify that the invention disclosed herein is the ☐ sole ☒ joint invention of the undersigned and that above statements and answers are true to the best knowledge and belief of the undersigned.

Date July 28, 1999	Signature John L. Hamm
Date July 28, 1999	Signature William F. Krise
Date	Signature

CERTIFICATION OF WITNESS(ES)

I certify that the invention described herein has been read and is understood by me.

Date July 28, 1999	Signature Kristine Playfoot	Post Office Address 18 Ross St. Mansfield PA 16933
Date	Signature	Post Office Address

V. SUMMARY RECORD OF THE INVENTIO[redacted]

Provide only the information requested [redacted] not submit records to which reference is made.

The making of an invention generally [redacted]es its conception followed by a series of acts which e[redacted]h the correctness or operativeness of the idea. Depending upon the nature of the invention, [redacted]ese acts may involve any one or all of the following: the making of sketches, drawings, written descriptions, the making and testing of a model, the carrying out of a process, or the production of a composition of matter.

A. CONCEPTION

Conception occurs when the essential elements of the invention in its operable and practicable form are fully disclosed in an oral description, in a written description, sketches, or drawings in such manner that the invention could be produced or practiced from them without the exercise of further inventive skill by a person who is skilled in the field to which the invention relates, and usually before the invention was physically tried out or produced in model form or a composition of matter was produced:

1. (a) Earliest date and the place where conception of the invention was made

06/21/89 For the over all invention and the fluid sample holder.
10/10/96 For the whole organism sample holder and new laser dyes.

(b) Earliest date and place this conception was related to others.

(c) Persons to whom disclosure of conception was made

To Kristine Playfoot, February 1999

2. Date and place of making the first sketch or drawing

Mansfield University

Present location of the first sketch or drawing, and identifying data for them (i.e., page number in a specified workbook)

3. Date and place of making the first written description

Present location of such description and identifying data for them (i.e., page number in a specified notebook)

B. REDUCTION TO PRACTICE

Reduction to practice occurs when a full scale working model or a prototype of the invention is made and operated as planned, or if the invention is a process, the process was tried out successfully, or if the invention is a composition of matter, the composition is actually produced.

1. (a) Date and place the first model or full size device was made, or process was first practiced or composition made

September 1998 to March 1999

(b) Identification of persons and/or records substantiating the facts indicated

Witnessed and corroborated by John L. Sternick and William F. Krise

2. (a) Date and place of the first successful operation or test of model, device, or process or composition

(b) Identification of persons and/or records substantiating the facts indicated

C. List other workbook entries, photographs, reports, correspondence, drawings, etc., that might have a bearing on supporting the conception and reduction to practice.

D. If the invention was disclosed to persons outside of the Department, identify the individuals, the companies or activities they represent, and the dates of such disclosures.

E. List with date the first known or contemplated (1) public use, (2) publication, or (3) oral presentation of the invention. "Public use" here means the use or practice of an invention for its intended purpose after testing or experimentation has shown that a workable form of the invention has been achieved.

VI. RIGHTS TO THE INVENTION.

Introduction.

A. Determination of rights:
Under Executive Order 10096, January 23, 1950, as amended by Executive Order 10930, March 24, 1961, and 43 CFR 6A, whenever an invention is made by an employee of the Department of the Interior, the employee may request a determination of the rights in the invention as between the Government and the inventor. There are three ways in which rights may be allocated: (1) the inventor may be entitled to all rights and the Government to none (in which event the inventor is not required to grant any rights to the Government); (2) the Government may be entitled to a license permitting it to practice the invention and the inventor entitled to all commercial rights (in which event the inventor is required to grant a license to the Government); (3) the Government may be entitled to all rights and the inventor to none (in which event the inventor is required to assign the invention to the Government).

B. Foreign filing:
Where the Government is entitled to all domestic rights in an invention it also acquired an option to secure foreign protection. The inventor will obtain the right to file in foreign countries if the Government determines not to exercise this option to file abroad, or permits the option to lapse as regards any foreign country by not filing or otherwise seeking protection of the invention within 6 months from the time a domestic application on the invention is filed.

C. Patent application filed by Government where inventor retains title:
Separate and distinct from the determination of rights, and even though it may appear that the inventor is entitled to all rights in the invention, the inventor may agree to license the Government to practice the invention in return for which the Government will prosecute an application for a patent on the invention at no expense to the inventor, provided the Government is sufficiently interested in the invention.

DESIGNATION BY INVENTOR IN RESPECT TO RIGHTS IN THE INVENTION

After carefully studying the provisions of 43 CFR 6.4, 6.5, 6.6 [1] the inventor should review the matter of his rights in the invention and indicate his wishes therein by selecting one of the options (1), (2), or (3), which follow.

(1) If the inventor is willing to voluntarily assign all rights (foreign and domestic) in the invention to the Government, he should place his signature, as indicated, after the following statement:
I am (We are) willing to assign to the United States Government the entire rights, title, and interest in and to the above-identified and described invention.

Date	*Signature(s) of the Inventor(s)*
_____	_____
_____	_____
_____	_____

(2) If the inventor is willing to assign to the Government the domestic rights only, and desires to retain foreign rights, he should place his signature as indicated, after the following statement:
I am (We are) willing to assign to the United States the entire domestic rights, title, and interest in and to the above-identified and described invention, and wish to retain the foreign rights thereto.

Date	*Signature(s) of the Inventor(s)*
_____	_____
_____	_____
_____	_____

(3) If the inventor is not willing to voluntarily assign at least all domestic rights in the invention to the Government, a determination of rights will be made as provided for in the orders and regulations identified above upon consideration of the information provided by the Invention Rights Questionnaire (Form No. DI-1218) attached hereto. Please check and sign below.

☐ A completed Invention Rights Questionnaire (Form No. DI-1218) is submitted herewith and a request for this determination is hereby made.

In the event the inventor retains title pursuant to such determination, the Government may file a patent application on the invention at its own expense for the inventor at his request if he agrees to grant the Government a royalty-free license to practice the invention for all governmental purposes, as explained in paragraph C above. Please check and sign below.

☐ I (We) request the Government to file under the conditions specified above.

Date	*Signature(s) of the Inventor(s)*
_____	_____
_____	_____
_____	_____

[1] Section 6.4 directs the Solicitor of the Department to determine, at the request of the employee-inventor, the respective rights of the Government and the employee in his invention.

Section 6.5 prescribes rules applicable in making the determination of rights in the invention. Under these rules the entire rights are obtained by the Government if the invention was made (a) during the employee's working hours, or (b) with a contribution by the Government of facilities, equipment, materials, funds, or information, or time or services of other Government employees on official duty, or (c) if the invention bears a direct relation to or is made in consequence of the official duties of the inventor. When the Government's contributions, as measured by the aforementioned criteria, are insufficient equitably to justify requiring the entire rights to go to the Government, or the Government has insufficient interest in the invention to obtain the entire rights (although it is entitled to the entire rights), the Solicitor, subject to the approval of the Commissioner of Patents in the Patent Office, is authorized to leave the title to the invention in the employee, subject to a reservation to the Government of an irrevocable, royalty-free license to the invention with power to grant sublicenses for all governmental purposes.

In applying the rules under section 6.5, there is a presumption that the invention belongs to the Government if the employee-inventor is assigned to invent or improve anything, to conduct or perform research or development work or both, to supervise, direct, coordinate, or review Government-sponsored research, development work, or both, or acts in a liaison capacity among Government or nongovernmental parties engaged in such work. When the work of the employee-inventor does not involve making inventions or improvements, research or development work, or both, as explained above, there is a presumption that title to the invention remains with the employee-inventor, and the Government is entitled to only a suitable license in the invention. However, section 6.5 also allows either presumption to be rebutted by a showing of the facts and circumstances in the case, and a determination that these facts and circumstances justify leaving the entire rights to the invention in the employee-inventor, subject to law. In any case where the Government neither obtains the entire domestic rights, or reserves a license, the Solicitor, subject to the approval of the Commissioner of Patents, is authorized to leave the entire rights to the invention in the employee-inventor, subject to law.

Section 6.6 provides for a procedure whereby any employee aggrieved by a determination of the Solicitor, may obtain a review of the determination by filing within 30 days after receiving notice of such determination, two copies of an appeal with the Commissioner of Patents. On a timely request by the employee appealing the Solicitor's determination, an oral hearing in the matter will be granted by the Commissioner of Patents. After the expiration of a time set for a reconsideration of the decision of the Commissioner of Patents, the decision becomes final.

* U.S. GOVERNMENT PRINTING OFFICE : 1971 O—425-991

NEAR INFRA RED MOLECULAR ASSAY

(NIRMA)

The necessity to develop a universal system for the detection of molecular species found in biological fluids or synthetic chemical environments has preoccupied and stimulated many scientists in academia and industry. The result of this labor was translated into a gamut of specialized instruments all of which are large, cumbersome, relatively slow, and expensive to operate, requiring a highly skilled staff.

Two of the most used techniques for measuring the presence and quantity of an analyte in a test sample are the ELISA and RIA.

There are many different types of ELISA procedures. The most general format consists of depositing the antigen of choice at a specific concentration in a 96 well plastic plate. The antigen solution is incubated in the plate for 1 hour before the excess antigen is washed out. The plate is now coated with antigen but the remaining electrostatic charges on the plate must be blocked with a protein buffer for another hour in order that test proteins and reagents (which are added later) do not non-specifically bind to the plate. The plate is washed once again before adding the test samples and incubated for another hour. The cycle of washing is repeated and an enzyme labeled anti-ligan is added and incubated for one more hour. Once this last incubation is finished, the plate is thoroughly washed and the enzyme substrate is added, if the enzyme is present, the substrate will be converted to product. A colorimetric change occurs which is measured with an ELISA machine. The data is then expressed on a computer screen or printed by a printer. In some ELISA's, the sample preparation may take a day before a reading is taken instead of 4 hours as in the above example.

An RIA is usually more sensitive than an ELISA. The probe is radioactive and requires special disposal facilities. The sequence steps of the assay are the same as the ELISA but the probe binds directly to the target molecule without enzymatic conversion of a substrate to a color product. Also, competitive RIA's are a common assay. Here, the radioactive probe and the non-radioactive molecule of interest found in the test sample compete for a common binding site. The gamma and beta radioactive counters are large table top or floor model instruments that also print out data.

Other techniques and complimentary instruments are used for the detection of biomolecules, some of which are: PANDEX, TDX, HPLC, PHAST, GC, FACS, and others.

In this paper the authors intend to describe a small handheld sensitive and fast analytical system which voids many of the shortcomings found in current technology. This new biosensor technology will be referred to as NIRMA. It can quantitatively and qualitatively determine, with the use of special reagents and disposables, the specificity and concentration of target molecules found in non-homogeneous fluids.

NIRMA is composed of 3 parts: 1) the hardware, 2) the reagents, and 3) the disposable reaction tips.

The Hardware

The device is an assembly of mechanical electro-optical and electronic systems that will now be described. For general outlay, see Fig. 1.

A small 0.5 W laser diode with an emission wavelength of 785 nm +/– 5 nm operating on its own 6 volt power supply is mechanically coupled to the sampler analyzer head (SAH) seen in Fig. 1, 2, 3. The SAH is a block of machined hard aluminum or

Teflon PTFE, which holds the optic housing, the bore for the disposable tip or tube and the negative/positive pressure connector. The optic housing containing a narrow band pass filter of 850 nm +/– 25 nm and a plano convex lens of BK7 glass. It is linked through a plastic, 0.95 mm diameter, fiber optic to a silicon photodiode-amplifier with a spectral peak of 740 nm +/– 50 nm which is wired to a LCD voltage display of 0.05% accuracy in 1 volt increments. The pressure connector of the SAH is attached to a 12 volt, 0.001 inch/step stepper motor syringe (piston) assembly with a short 18 gauge tygon tube. The electrical power for the stepper motor is activated by a motor control board with a variable speed control switch. The total system is synchronized by a master power and electronic timer control board. The photodiode can be replaced by a photon counting module for more sensitive emission measurements. The level of fluid in the reservoir and the uptake channel is determined by timers or by two miniature sensors (sonic) in the SAH which are connected to the stepper motor board, see Fig. 3.

A second prototype seen in Fig. 4 is the Eel Laser Instrument. In this device, the SAH and the detector are in one block, which is made up of a 4.25 mW light source, and a photodiode detector wand that attaches to a hand-held optical power meter. The wand does not need a narrow band pass filter set before its detector window, since the wand is calibrated to measure only light emitted at 806 nm.

The detector in the Eel Laser Instrument measures the light emitted from the laser dye in the fish in microWatts (μW). An increase in the μW readings corresponds to an increase in the amount of light emitted and hence corresponds to a set amount of laser dye (NN 382) in the sample or fish tested.

The Eel Laser Instrument has an eel sample tube channel (see Fig. 4) that can have varying diameter to accommodate various size fish. The eel sample tube channel is manufactured with a large diameter that can be reduced by inserting a series of tubes with smaller internal diameters, to accommodate the correct sample size.

Eels passing through the sample tube channel can be counted by breaking a light path (electric eye) as they flow through the channel. The electric eye is placed at the entrance of the SAH. Eels flowing through the tubing can activate the Eel Laser Instrument.

The Reagents

It is evident that from among all the spectrofluorescent methods, laser fluorimetry is the most sensitive (1-6). Sauda et al. (1986) demonstrated detection of 10^{-12} M laser dye with a 3 nW laser. Also, there are over 2000 laser dyes commercially available (7) from which an inventor must chose in order to develop a practical detection system. Therefore, a diode laser, a photodiode, and a laser dye must match in order to make the detection system workable.

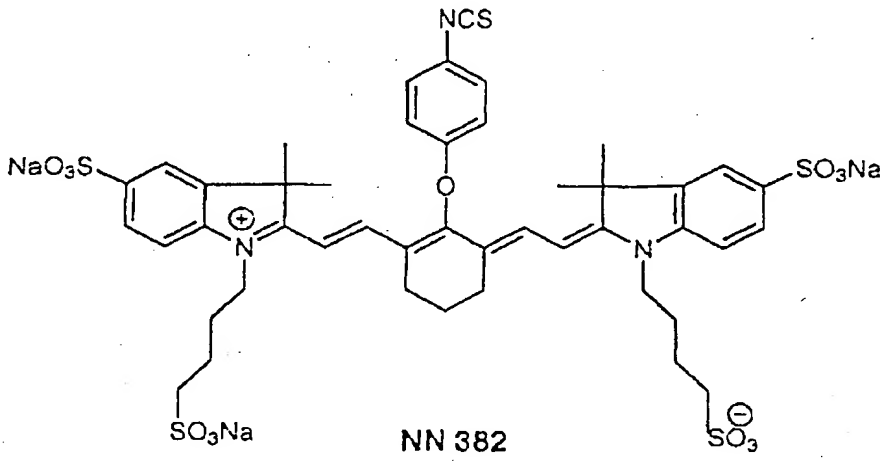
Since most biological and other materials do not fluoresce between 700 and 1300 nm (4) it was important to find a useful laser dye in that range which could bind to a specific carrier or labeling molecules and act as a tag, without loosing its fluorescent properties.

It appears that polymethine dyes have an absorption range between 600 and 900 nm (4) but most are positively charged and not very useful for directly labeling proteins. Some, such as IR-125 and IR-144 are negatively charged and hence ideal for protein

labeling (8). A new dye, NN 382, can also be used since it has good labeling characteristics.

The laser dye selected for NIRMA is NN 382. Its characteristics are as follows: 1) absorption maximum is 778 nm, 2) emission maximum is 806 nm, 3) soluble in DMSO and related organic solvents, 4) soluble in pure water, 5) polymerizes and quenches at high concentrations, 6) non toxic, 7) can be stored frozen for long term storage, 8) stable when coupled to proteins and stored in the cold (below 5° C), and 9) binds electrostatically to specific proteins such as albumin, lipoproteins and gamma globulins. The structure is shown below, its molecular formula is:

$C_{45}H_{48}N_3O_{13}S_5N2_3$ with a M • W of 1067



NN 382

The principle of detection is to couple NN382 to a labeling molecule such as an antibody, specific ligans like avidin, protein A, protein G, various antigens or solid materials, i.e.: ion exchange resins, latex beads or other inert non-fluorescent structures and use this labeled compound as the reagent tag, i.e.:

NN382 + LABELING MOLECULE or SOLID MATERIAL = REAGENT TAG

The standard detection techniques are then carried out as described in the following diagrams 1 through 5; diagram 6 is a special application. In diagram 6, the detection is accomplished without the interference of any immobilizing matrix or reaction vessel wall. This special detection system eliminates any background emission from materials other than the tag and hence is more sensitive than the matrix dependent method. By lowering the background to near zero, the measurements are more accurate and precise since they do not depend on the quality of materials used in the production of disposable tips. The results are expressed in positive values instead of the negative correlations seen in competitive RIA's. In a competitive RIA system, the amount of radioactive label seen or counted decreases as the target molecule being detected increases. In the NIRMA, an increase in level or concentration of the target molecule directly corresponds to an increase of NN382, hence an increase in light emission.

The Disposable Reaction Tips

There are two fundamental disposable tip configurations, which are defined by two different types of analysis target area (ATA). The first type (type 1) has an enclosed ATA composed of a solid phase and the second type (type 2) has an ATA which is solid phase free and not enclosed by materials, as seen in Figs. 5 and 6, respectively. The quantity of bound or free probe (depending on the tip used) is excited and its emission quantified.

In tip type 1, Fig. 5, the material used for the wall of the disposable tip has a low near infra red excitation/emission profile in the wavelength of interest as mentioned previously. This material is Teflon FEP, other materials are suitable but have higher near infra red background or other less appealing characteristics such as carrying larger

electrostatic charges on their surface and hence attracting and binding biomolecules of interest or reagent tag which renders the assay less sensitive. The tip's electrostatic charges must be specially blocked without interfering with the binding characteristics of the plug or matrix. Also, since the tips can be used in a sterile environment, they must be sterilizable. This must be done preferably by radiation, and must not change the properties of FEP or any other materials used in the construction of these tips, so that the assay is not jeopardized in any way.

The ATA can be made of micro ground glass, micro glass, or plastic beads as well as porous nylon matrix, porous composite matrix (for example: nylon and ion exchange resins) or a fine mesh or screen surface. These materials have the same near infra red, low non-specific binding and sterilizable properties as the FEP housing above. Also, they have an added characteristic, they can bind covalently or by other means "target" or "capture" molecules used in the NIRMA system. The "target" molecules are the chemical structures which are bound to the matrix for the purpose of having specific molecules recognize and bind to them when they are in very close proximity, for example: the target molecule could be an antigen, i.e.: an antibody, a ligan such as avidin, concanabilin A, protein A and G, etc., or a hapten, i.e.: biotin, an enzyme substrate or its product, a chelate, etc. The matrix may not carry any "target" or "capture" molecules at first but may be supplied in an activated form so that the user can attach any ligan of choice to the matrix.

In Fig. 5A, an FEP tube (24 gauge) is shown with a plug composed of activated ground glass sandwiched by two plastic porous (porex) discs. The tube, which is a prototype of the disposable tip, fits into a pressure fitting with an "O" ring to seal the

tube. In Fig. 5B, the reservoir is larger than the uptake channel permitting a bigger sample volume to be collected and tested. Also, an internal pressure fitting is shown versus an external one with an "O" ring in Fig. 5C.

In Fig. 6A, the type 2 disposable tip has the same external disposition as the tip in Fig. 5B, except for a double window in front and in back of the ATA. Internally, type 2 tip has a small bore tube specially designed to bring a small miroliter bubble in front of the detection window. The tube end is designed so as not to touch the internal wall of the tip. Also, by beveling the tube one increases the surface area for better surface adherence of the bubble.

The design is modified in Fig. 6B to flatten out the bubble in which the detection takes place; a post is set close to the tube end. In this last case, the bubble is hung between the tube end and the post, hence increasing the stability of the sample and making surface readings possible with less scatter of the emission wavelength from the reagent tag. A space exists on each side of the post (between the post and the internal wall of the tip) so that air has access to the uptake channel. When a negative pressure is created, the fluid sample can rise through the uptake channel into the reagent trap and past it to the ATA.

The type 2 disposable tips have a "trap" which is a zone where two competing molecules bind to a matrix. The trap is situated before the end of the specially beveled tube as shown in Fig. 6A and B.

Methods of Operation

In the first case, an antibody determination is carried out as follows (see Fig. 7): 1) a disposable tip (type 1) has a specific antigen Z1 bound to its matrix. This is done by

preferably using covalent coupling chemistries; 2) a fluid sample containing the specific antibody "Z" to antigen "Z1" is aspirated through the uptake channel past the matrix. The antibody "Z" binds to the antigen "Z1"; 3) the sample fluid is stopped at a predetermined level inside the disposable tip so as not to contaminate the SAH; 4) the sample is then expelled from the tip. The antibody "Z" is still binding to the antigen "Z1" during this phase as long as there is more antibody "Z" available for binding and the antigen "Z1" is not saturated by an initially high concentration of antibody "Z"; 5) a wash buffer is then flowed through the matrix to wash off the sample. This is done as in 3 and 4 above; 6) this is followed by the uptake of the specific reagent tag to a determined level past the matrix. The reagent tag is in this case an antibody tag binding to antibody "Z" to which it binds if present. The amount of antibody tag binding to antibody "Z" determines the intensity of the emission. If no antibody "Z" is present in the sample, no antibody "Z" will bind to antigen "Z1" and hence no antibody tag will be in the ATA since there are no antibody "Z" to bind to; 7) the reagent tag is expelled; 8) the unbound reagent tag is flushed out of the matrix as in 3 and 4 above, but the matrix is kept wet (this enhances the reading); 9) the diode laser is fired for a predetermined amount of time and the photodiode picks up the quantity of light emitted by the reagent tag; and 10; the voltage generated is amplified and displayed on the liquid crystal display (LCD). This number represents a known quantity of antibody "Z". The molecular binding sequence of events is represented in diagram 1, 2, and 4a.

In diagram 4a, the reagent tag is not an antibody but an avidin tag binding to a biotinilated antibody, which is attached to a specific antibody to the antigen (Ag 2) coupled to the solid matrix.

In the second case, an antigen determination is carried out. The mechanical sequence of events is identical to those of the first case. The molecular interactions between antibody are bound to the matrix instead of the antigen. This can be accomplished by direct binding of the antibody to the matrix or through linkers and spacers. One form of a spacer molecule is avidin. The coupling of avidin to the matrix followed by the binding of a specific biotinilated antibody "Xa" as the capturing molecule represents a universal coupling technique useful in antigen detection. This antibody "Xa" recognizes and captures antigen "X2" which is then recognized and bound by a second antibody reagent tag "Xb". Diagram 3 illustrates multiple variations of this theme.

In diagram 4b, the antigen (Ag 1) is captured by the matrix bound Ag 1 specific antibody. The antigen is then recognized and bound by a second biotinilated antibody, which is then bound by the avidin reagent tag.

The antibody or antigen determinations can also be made by reagent tags composed of near infra red probe in or on latex particles, ion exchange resins or other particulate matter with the appropriate biophysical and chemical characteristics (Diagram 5).

The detection of emitted light from the reagent tag is shown in Fig. 2. The laser diode excites NN 382 at 785 nm, which is very close to the maximum excitation absorbance of the laser dye, i.e.: 778 nm. The laser light hits the matrix in the disposable tip at a 45 degree angle but the detector lens and filter are at right angle to the emitting matrix. It is important to note that the matrix is not transparent and therefore the detection of light must be on the same side as the excitation in order to capture most of

the signal from the matrix. Also, the excitation angle prevents most of the reflected light from entering the detection system since it bounces off at a 45 degree angle away from the lens. The filter stops the laser light from entering the optical housing and only lets the emission light pass through. The emitted light from the NN 382 on the matrix is focused on the end of a fiber optic by a plano-convex lens closely situated to the emission source. The closer the lens is to the source the more light it can capture, hence a large lens with a short focal length would be ideal but if the optical housing gets too close to the emission source it infringes on the laser light path. The optical housing and the SAH chambers are coated with a black mat non reflecting or emitting paint.

The above assays are based on type 1 disposable tips where the reagent tag is on a matrix surrounded by the tip wall. The assay described below is based on type 2 disposable tips, which increases the sensitivity and decreases the error of the assay.

With the type 2 tip, the matrix has a ligan coupled to it which specifically binds the molecule of interest in a test sample as well as the reagent tag, which is the same as the molecule of interest but labeled with NN 382 (this is a competitive assay), i.e.: an antibody "b" coupled to the matrix specifically recognizes and binds the test sample antigen "b1" flowing through it as well as the reagent tag (antigen "b1-NN 382"). In order to measure the amount of antigen "b1" in a test sample; a calibrated quantity of reagent tag is mixed with the test sample. The amount of reagent tag added to the test sample is just enough to saturate all the matrix bound antibody "b" binding sites when no other competitive antigen to the reagent tag is present in the sample being tested. Hence no excess reagent tag goes through the matrix (its all captured by the antibody on the matrix) and appears in the ATA, giving a zero reading. The only means by which the

reagent tag appears in the ATA is when the reagent tag and antigen "b1" from the test sample are mixed. The test sample antigen "b1" competes against the reagent tag for the same binding site of antibody "b" located on the matrix and therefore some of the reagent tag is not bound to the matrix. The free reagent tag flows into the ATA where it's measured. In diagram 6, one can observe that as increasing amounts of test sample antigen are present in the mixture more and more of the reagent tag appears in the ATA. This method shows that with a very low concentration of test sample antigen "b1" one gets low readings while with equal amounts of reagent and test antigen "b1" half of the reagent will appear in the ATA bubble and finally when high concentrations of antigen "b1" are being tested most of the reagent tag will be found in the ATA. Graphic representation of the NIRMA versus RIA is shown as the last part of diagram 6. In the NIRMA graph, one can see that as the concentration of antigen in the sample rises, the reagent tag readings increase proportionately. In the standard competitive RIA, the converse is true, as the concentration of the antigen in the sample increases, the amount of radioactive tag being measured decreases. The RIA requires the generation of a standard curve while NIRMA does not since it's established that a certain amount of antigen binding the matrix will displace an equal amount of reagent into the ATA.

The details of the measurements taken in the ATA of the disposable tip 2 can be seen in Fig. 6c. The exciting laser light is focused in the center of the bubble which is the ATA, while the emission wavelength from the bubble is captured by the detector lens without any interference from building materials used in making the tip.

Finally, when using the eel laser instrument, the eel is passed through the eel sample tube channel, Fig. 4. The eel is aligned with the ATA, the laser fired, and the

amount of NN 382 in the eel measured from its emission by the optical detector wand. The results are expressed in mW.

Advantages of NIRMA

In summary this detection system brings a new level of technology to the user. The practical aspect of NIRMA are tabulated below:

1) Speed of operation, seconds versus hours or days.
2) Small sample size, from less than a microliter to many microliters.
3) Non-Isotopic assay.
4) Small tool, portable, hand held, and is designed to be battery operated.
5) No fluorescent background because of the selection of the NIR range of the reagent tag.
6) High sensitivity of the assay because of the NIR, two types of tips and electro-optic designs.
7) No precalibration or standard curve necessary.
8) Sturdy tool.
9) Little training is necessary to instruct the operator, it's an intuitive system.
10) Can be used under sterile and non-sterile conditions.
11) Non-enzymatic assay.
12) Can be adapted to low concentrations of molecules in test sample by selecting tip and optical system.

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